Biochemistry of cholesterol

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Prefa

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Significance of rate-limiting steps

In a chain of biochemical reactions each catalysed by a separate enzyme the overall rate of conversion of the primary substrate into the end product will be determined by the reaction with the lowest velocity. This reaction is called the rate-limiting step. Any factor which modifies the capacity of this step also modifies proportionately the overall rate of synthesis of the final product, provided that the step continues to be rate-limiting. The concept of the rate-limiting step is rather theoretical since it is a matter of common experience that most metabolic sequences operate at a rate considerably less than the maximum; this is evident in higher organisms as well as in unicellular organisms. For example, in yeast, glycolysis proceeds at less than the maximum rate in the presence of air (the Pasteur effect) and in higher animals the respiratory rate of skeletal muscle at rest is a small fraction of the rate of working muscle. Moreover, the maximum velocity of a particular step measured in vitro under optimal conditions of assay is not necessarily the velocity at which that step operates in vivo. It is also obvious that what is considered optimal for a particular step may not be for other steps in the sequence. In view of these uncertainties evidence for the importance of a particular step in the control of the rate of synthesis of the final product is indirect, identification of the rate-limiting step being established as a result of evidence derived from different sources.

The presence of a rate-limiting step in a biosynthetic pathway simplifies the study of its regulation. Moreover, investigation of the properties of the enzyme catalysing this step may shed light on the molecular mechanism involved in the regulation of the overall pathway. A rate-limiting step is usually an irreversible reaction and is often the first step after a branch in a biosynthetic sequence. The evidence for the role of hydroxymethylglutaryl-CoA reductase as the rate-limiting enzyme for the overall rate of cholesterogenesis is considered in the next section; that of cholesterol 7α-hydroxylase in bile-acid synthesis is considered in the subsequent section.

Regulation of cholesterol biosynthesis

The rate-limiting step in cholesterol biosynthesis

The discovery that [2-^{14}C]mevalonate is converted into cholesterol by rat liver subcellular fractions with high efficiency (Tavormina et al., 1956) suggested that the steps beyond mevalonic acid are not rate-limiting. The investigation of the effects of dietary, hormonal or environmental factors on the rate of incorporation of radioactivity from acetate or from mevalonate into cholesterol by liver subcellular fractions has shown that the step that is regulated is earlier than mevalonic acid (Gould and Popják, 1957) and, by inference, after hydroxymethylglutaryl-CoA (Bucher et al., 1960; Siperstein and Fagan, 1966). This inference was based on the following argument. Acetoacetyl-CoA is the first intermediate of both cholesterol synthesis and ketone body formation in the liver (Bucher et al., 1960; Siperstein, 1970). Since dietary cholesterol suppresses hepatic cholesterogenesis from acetate but not the synthesis of ketone

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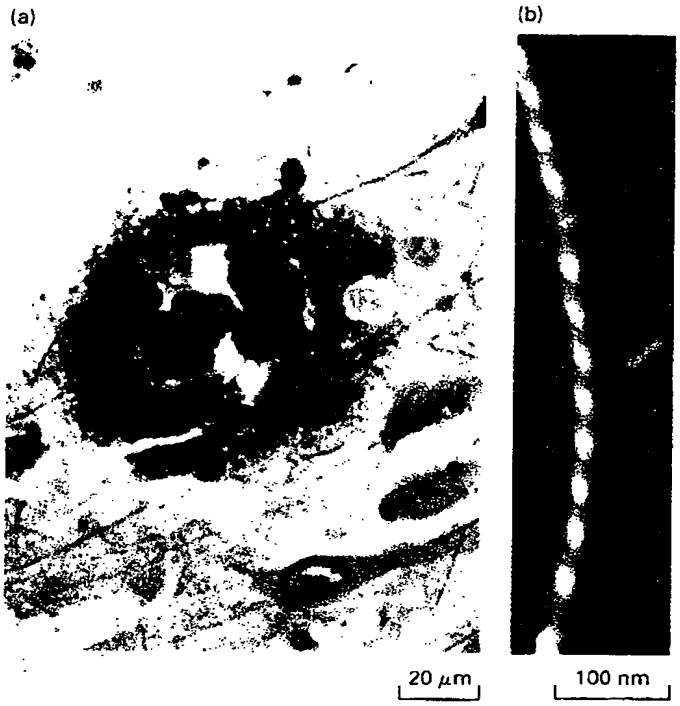
bodies from the same substrate, it was thought that hydroxymethylglutaryl-CoA is the branch-point for the two pathways. It was therefore concluded that hydroxymethylglutaryl-CoA reductase is the first enzyme 'unique' to cholesterogenesis. Moreover, the irreversibility of the reaction catalysed by hydroxymethylglutaryl-CoA reductase (Bucher et al., 1960) satisfied the criterion for a typical regulatory site. However, we now know that ketogenesis via acetoacetyl-CoA is a mitochondrial process (Garland, 1968; Greville and Tubbs; 1968), whereas cholesterol synthesis from this precursor occurs extramitochondrially. Recent investigations have revealed that substantial activities of acetoacetyl-CoA thiolase and hydroxymethylglutaryl-CoA synthase are present in the cytosolic fraction of avian (Sugiyama et al., 1972) and mammalian (Middleton, 1973) liver and that these enzymes are capable of efficiently converting acetyl-CoA to hydroxymethylglutaryl-CoA. For an account of the metabolic source of acetyl-CoA in cholesterol biosynthesis see also p. 141. Although a number of assumptions underlying the conclusion that hydroxymethylglutaryl-CoA reductase is the rate-limiting enzyme for cholesterogenesis have been shown to be invalid, the conclusion itself is valid, since many observations have shown that the activity of this enzyme and the rate of synthesis of cholesterol in the liver change in parallel when enzyme capacity is increased or decreased. This is exemplified by the observations of Dietschy and Brown (1974) showing that reductase activity measured in rat liver microsomal fraction and expressed as the C_2 flux into mevalonic acid correlates closely with the C_2 flux into cholesterol from [1-^{14}C]octanoate catalysed by liver slices from the same rats (Fig. 8.1). This correlation holds over a wide range of cholesterol synthetic capacity achieved by dietary and other manipulations. For reviews of the regulatory role of hydroxymethylglutaryl-CoA reductase in cholesterol biosynthesis and of the properties of the enzyme the reader is referred to Rodwell et al. (1973, 1976).

Other regulated steps in the biosynthesis of cholesterol

Acetoacetyl-CoA thiolase, a ubiquitous enzyme in animal tissues, functions in three metabolic pathways in liver. Two of these, ketogenesis and β-oxidation, are exclusively localised in the mitochondrion, whereas cholesterogenesis is confined to the extramitochondrial space. Hydroxymethylglutaryl-CoA that is destined to be incorporated into cholesterol is synthesised in the cytoplasm by the enzymes acetoacetyl-CoA thiolase and hydroxymethylglutaryl-CoA synthase (Middleton, 1972; Clinkenbeard et al., 1973). Since the conversion of acetyl-CoA to hydroxymethylglutaryl-CoA is a thermodynamically favourable process (Sugiyama et al., 1972), control of the thiolase and synthase activity might be expected in order to prevent unnecessary depletion of the cytoplasmic acetyl-CoA pool when cholesterogenesis is drastically reduced.

Lane and his colleagues (Clinkenbeard et al., 1973) found that feeding a diet containing 2% of cholesterol to chickens for 1 week causes a drastic reduction in the activity of liver cytosolic hydroxymethylglutaryl-CoA synthase and a significant decrease in the cytosolic acetoacetyl-CoA thiolase activity (Sugiyama et al., 1972).



▲ **FIGURE 3-19 Amyloidosis is characterized by the formation of insoluble protein plaques in various organs of the body.** (a) The amyloid plaque in the brain of an Alzheimer's patient appears as a tangle of filaments. (b) In the atomic force microscope, the filaments are seen to be regular arrangements of a short 47-residue fragment, called *β-amyloid peptide*, produced by proteolysis of amyloid precursor protein. [Courtesy of K. Kosik.]

the brain, similar to that seen in Alzheimer's disease, is thought to be caused by *prions*, an infectious protein agent derived by proteolysis and re-folding of a normal brain protein.

SUMMARY Folding, Modification, and Degradation of Proteins

- The amino acid sequence of a protein dictates its folding into a specific three-dimensional conformation, the native state.
- Folding of denatured proteins in vitro proceeds through intermediates having secondary and non-native tertiary structure.
- Protein folding in vivo occurs with the assistance of two types of special proteins (see Figure 3-15). Molecular chaperones (Hsp70 proteins) bind to nascent polypeptides emerging from ribosomes and prevent their misfolding. Chaperonins, large complexes of Hsp60-like proteins, shelter some partially folded or misfolded proteins in a barrel-like cavity, providing additional time for proper folding.
- Following their synthesis, all proteins are modified in various ways that alter their structure and function.
- The life span of intracellular proteins is largely determined by their susceptibility to proteolytic degradation by various pathways.
- The presence of certain internal sequences or N-terminal residues targets cytosolic proteins for addition of ubiquitin and subsequent proteolysis within a proteasome (see Figure 3-18).

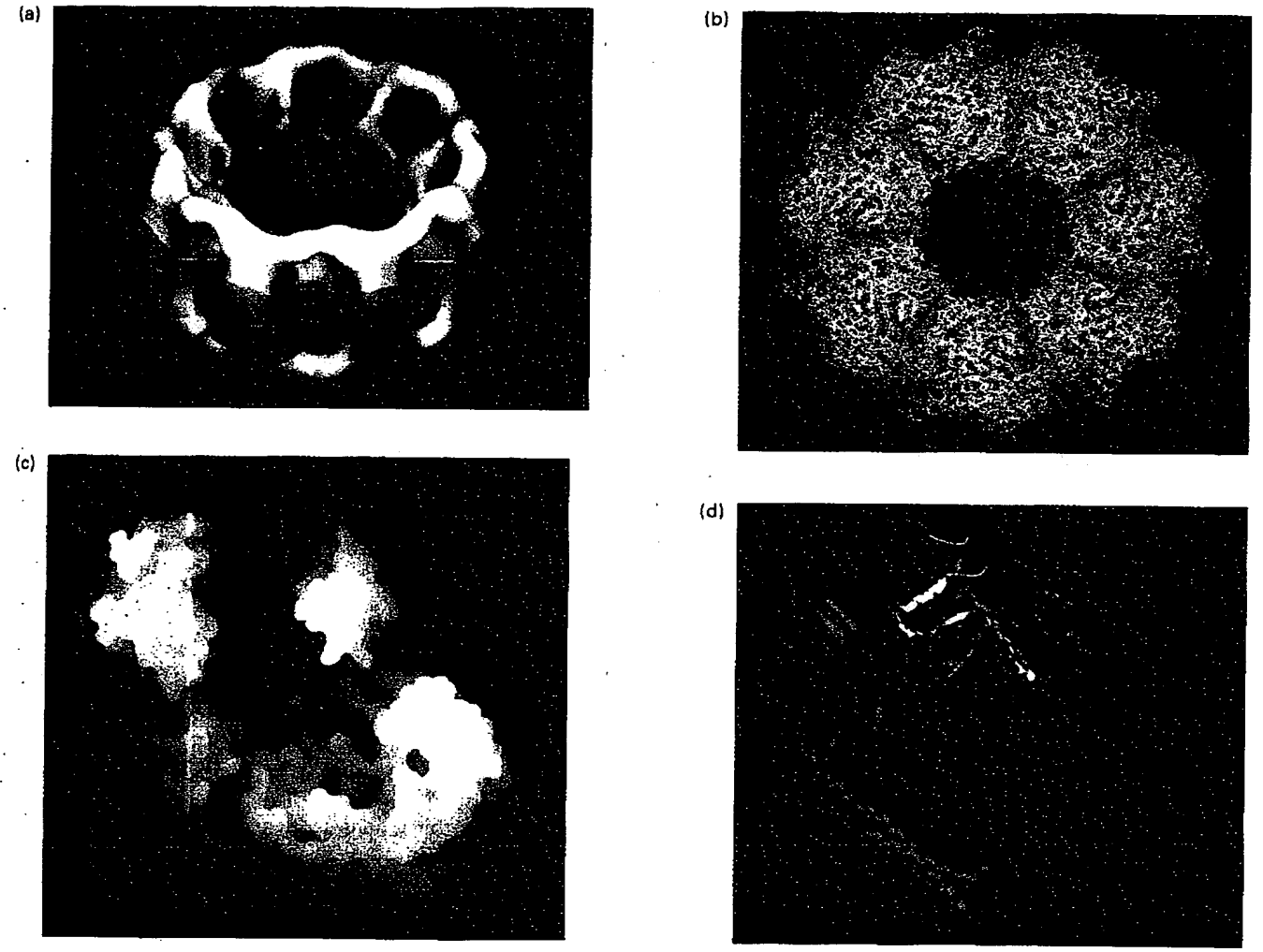
3.3 Functional Design of Proteins

A key concept in biology is that form and function are inseparable. This concept applies equally well to protein design as to other levels of biological organization (e.g., the morphology of cells and the organization of tissues). In fact, we can often guess how a protein works by looking at its structure. Perhaps the best way to illustrate this is by examining a few protein structures. For instance, a barrel-like nuclear pore, a complex of several proteins, sits in the nuclear membrane and acts as a channel through which molecules travel in or out of the nucleus (Figure 3-20a). In the cavity of a different barrel-like structure, the GroEL/ES chaperonin, protein folding takes place (Figure 3-20b). Some proteins have grooves in their surface, which are logical binding sites for a variety of molecules, especially rod-shaped or filamentous ones. An example is reverse transcriptase, which copies RNA into DNA; this enzyme has a groove on one side through which RNA slides along the surface of the protein (Figure 3-20c). Topoisomerase II, a DNA-binding enzyme, is an articulated enzyme that opens and closes at both ends like locks in a canal (Figure 3-20d). A delight in studying protein structure is uncovering the simple but ingenious ways that nature has built each protein to perform a particular function.

In this section, we examine several features of proteins that are critical to their biological activity and the regulation of that activity, focusing on antibodies, enzymes, and membrane proteins as examples. The functioning of many proteins involves some change in their conformation induced by binding of a specific molecule, change in the environment, or chemical modification. As numerous examples in later chapters will illustrate, such induced conformational changes can make proteins into switches and machines. The changes in conformation can be enormous, as seen in proteins like topoisomerase, an enzyme that moves DNA strands across one another, or myosin, a motor protein that moves along actin filaments.

Proteins Are Designed to Bind a Wide Range of Molecules

The function of nearly all proteins depends on their ability to bind other molecules, or **ligands**, with a high degree of specificity. As catalysts of chemical reactions, enzymes must



▲ **FIGURE 3-20 Gallery of protein structural models showing the link between structure and function.** (a) The nuclear pore, a complex of proteins with a total molecular weight of 1.2×10^8, an outer diameter of 133 nm, a height of 70 nm, and a central hole 42 nm in diameter. Transport of molecules into and out of the nucleus occurs through such pores located in the nuclear membrane. (b) Protein folding takes place within the cavity of a chaperonin, GroEL/ES. Built of 21 subunits, the chaperonin is 18.4 nm long and has a diameter of 3.3 nm, large enough for small- and medium-sized proteins. (c) Reverse transcriptase, an enzyme present in RNA viruses that copies the viral RNA genome into DNA. Reverse transcriptase from the AIDS-causing virus HIV measures 11 × 3.0 × 4.5 nm. RNA to be copied lies in a groove on the surface of the enzyme. (d) Topoisomerase II, which prevents DNA from overtwisting during replication, is shaped like a pair of tongs. Opening and closing of Topo II at the top and bottom of the protein permit a nicked strand of DNA to pass through and be repaired. [Part (a) from J. E. Hinshaw, B. O. Carragher, and R. A. Milligan, 1992, *Cell* **69**:1133; part (b) courtesy of S. Choe; part (c) from T. A. Steitz et al., 1992, *Science* **256**:1783; part (d) courtesy of J. Berger.]

first bind tightly and specifically to their target molecules, called **substrates**, which may be a small molecule (e.g., glucose) or a macromolecule. The many different types of hormone receptors on the surface of cells also display a high degree of sensitivity and discrimination for their ligands, which generally are present at low concentrations in blood. These receptors, essential to signaling between cells, are discussed in Chapter 20.

Two properties of a protein characterize its interaction with ligands. *Affinity* refers to the strength of binding between a protein and ligand; the equilibrium constant K_{eq} (Chapter 2) or the dissociation constant K_D for binding is a measure of affinity. *Specificity* refers to the ability of a protein to bind one molecule in preference to other molecules. Both properties depend on the structure of the ligand-binding site on a protein, which is designed to fit its partner

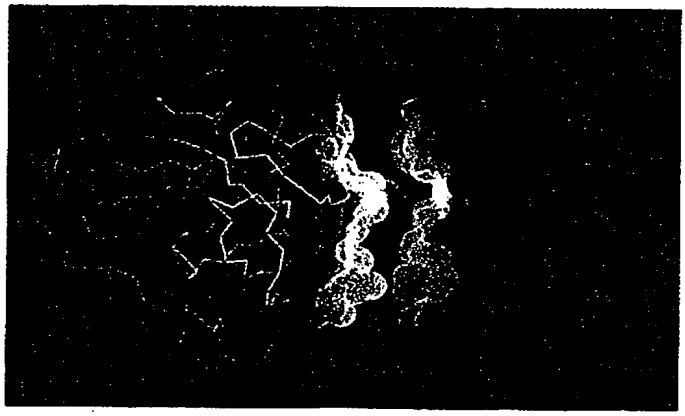
like a mold. For high-affinity and highly specific interactions to occur, the shape and chemical surface of the binding site must be complementary to the ligand molecule. To illustrate this critical concept, we consider how an antibody binds an antigen and how an enzyme catalyzes a chemical reaction.

Antibodies Exhibit Precise Ligand-Binding Specificity

The capacity of proteins to distinguish different molecules is highly developed in blood proteins called **antibodies.** Animals produce antibodies in response to the invasion of an infectious agent (e.g., a bacterium or a virus) or after exposure to certain foreign substances (e.g., proteins or polysaccharides in pollens). The antibody-inducing agent is called an **antigen.** The presence of antigen causes an organism to make a large quantity of different antibody proteins, each of which may bind to a slightly different region of the antigen. The constellation of antibodies induced by a given antigen may differ from one member of a species to another.

All antibodies belong to a family of proteins called **immunoglobulins.** These Y-shaped molecules are formed from two types of polypeptides: heavy chains and light chains. The heavy chains run the length of the molecule; their C-terminal regions pair to form a stem. Visually we can distinguish three globular domains: two identical domains corresponding to each arm and the third composing the stem (Figure 3-21). Each arm of the antibody molecule contains a single light chain linked to a heavy chain by disulfide bonds. The N-terminal regions of both heavy and light chains lie at the tip of each arm and are distinguished by highly variable amino acid sequences. The remaining portions of the sequences in both chains are constant (i.e., nearly identical) among antibodies with different specificities. The arms are the business end of an antibody molecule, since an antigen-binding site lies at the end of each arm. Because of its dimeric structure, each antibody molecule can bind two identical antigen molecules. X-ray crystallographic analysis of antigen-antibody complexes has revealed that the antigenic specificity of an antibody is dependent on three highly variable regions, called *complementarity-determining regions (CDRs)*, near the end of each arm. These regions form the antigen-binding site, which physically matches the antigen like a glove.

▲ **FIGURE 3-21 Structure of an antibody molecule, which consists of two identical heavy chains (blue and orange) and two identical light chains (yellow and green).** The Y-shaped molecule contains two identical Fab domains, forming the arms, and one Fc domain, forming the stem. In the native molecule, each heavy chain is a continuous polypeptide, with a hinge region connecting the two halves shown in this figure. Antigen molecules (white) bind to the complementarity-determining regions (CDRs), which are highly variable regions located at the ends of each arm. Antibodies contain carbohydrate moieties (red) and thus are glycoproteins. [From A. Levine, 1992, *Viruses*, W. H. Freeman, p. 53.]



▲ **FIGURE 3-22 The hand-in-glove fit of an antibody *(right)* to an antigen from influenza virus *(left)*.** This computer simulation is based on x-ray crystallography of the complex formed between the antigen and the antibody Fab domain. The complementarity of the antigen and antibody surfaces is especially apparent where the "finger" extending from the antigen surface is opposed to the "cleft" in the antibody surface. [From G. J. V. H. Nossal, 1993, *Sci. Am.* **269** (Sept.):54.]

Most large antigens have multiple different sites, called **epitopes** (or antigenic determinants) that can induce production of specific antibodies; each type of antibody binds to its own inducing epitope. For example, lysozyme, an enzyme that degrades the carbohydrate coat of bacteria, induces several different antibodies, each of which binds to a particular epitope on the lysozyme molecule. Although the different epitopes on lysozyme differ greatly in their chemical properties, the interaction between lysozyme and antibody is complementary in all cases; that is, the surface of the antibody's antigen-binding site fits into that of the corresponding epitope as if they were molded together (Figure 3-22). The intimate contact between these two surfaces,

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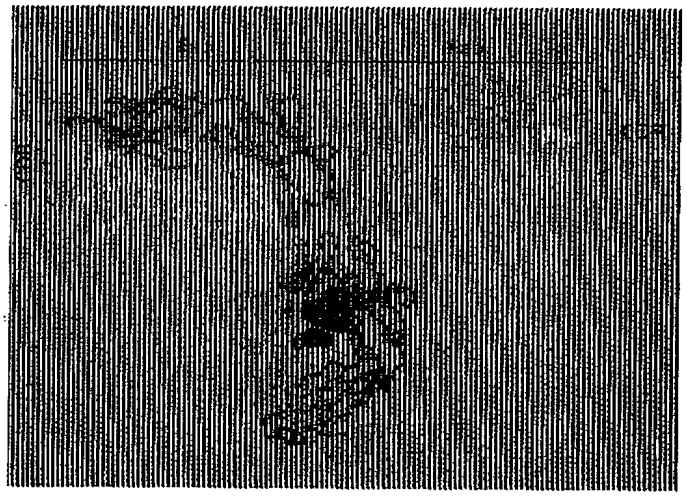

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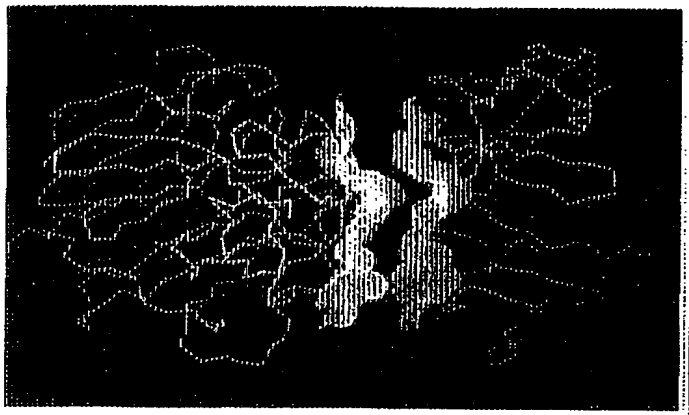

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stabilized by numerous noncovalent bonds, is responsible for the exquisite binding specificity exhibited by an antibody. Antibodies, for instance, can distinguish between the cells of individual members of a species and in some cases can distinguish between proteins that differ by only a single amino acid. Because of their specificity and the ease with which they can be produced, antibodies are critical reagents in many experiments discussed in the following chapters.

Enzymes Are Highly Efficient and Specific Catalysts

Almost every chemical reaction in a cell is catalyzed by a class of proteins called **enzymes.** As discussed in Chapter 2, catalysts increase the rates of reactions that are already energetically favorable by lowering the activation energy (see Figure 2-27). In the test tube, catalysts such as charcoal and platinum facilitate reactions but often at high temperatures, at extremes of high or low pH, or in organic solvents. In contrast to these harsh conditions, enzymes must catalyze chemical reactions in the mild conditions of a cell: 37 °C, pH 6.5–7.5, and aqueous solvents. As we just discussed, all antibodies belong to the immunoglobulin family of proteins and have a similar structure. Enzymes, however, are a structurally diverse group of proteins that have evolved through unrelated and highly divergent mechanisms.

The ability of enzymes to function as catalysts under conditions where nonbiological catalysts would be ineffectual is exemplified by two striking properties: their enormous reaction rates and their specificity. Quite often, the rate of an enzymatically catalyzed reaction is 10^6–10^{12} times that of an uncatalyzed reaction under otherwise similar conditions. The specificity of an enzyme denotes its ability to act selectively on one substance or a small number of chemically similar substances, the enzyme's substrates. Like antibody specificity, enzyme specificity depends on a close fit between substrate molecules and their binding sites on an enzyme. An example of specificity is provided by the enzymes that act on amino acids. As noted in Chapter 2, amino acids can exist as two stereoisomers, designated L and D, although only L isomers normally are found in biological systems. Not surprisingly, enzyme-catalyzed reactions involving L-amino acids occur much more rapidly than do those involving D-amino acids, even though both stereoisomers of a given amino acid are the same size and possess the same R groups (see Figure 2-6).

The number of different types of chemical reactions that occur in any one cell is very large: an animal cell, for example, normally contains 1000–4000 different types of enzymes, each of which catalyzes a single chemical reaction or set of closely related reactions. Certain enzymes are found in the majority of cells because they catalyze synthesis of common cellular products (e.g., proteins, nucleic acids, and phospholipids) or are involved in the production of energy by the conversion of glucose and oxygen to carbon dioxide and water. Other enzymes are present only in a particular type of cell (e.g., a liver cell or a nerve cell) because they catalyze some chemical reaction unique to that cell type. Although most enzymes are located within cells, some are secreted and function in the blood, lumen of the digestive tract, or other extracellular space. Some microbial enzymes are secreted from and are active outside the organism.

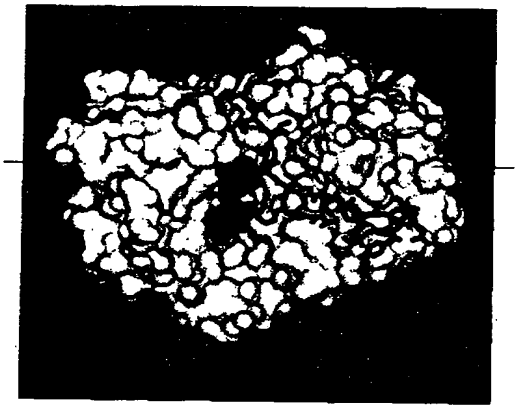
An Enzyme's Active Site Binds Substrates and Carries Out Catalysis

Certain amino acid side chains of an enzyme are important in determining its specificity and its ability to accelerate a chemical reaction. In the native conformation of an enzyme, these side chains are brought into proximity, forming the active site. Active sites thus consist of two functionally important regions: one that recognizes and binds the substrate (or substrates), and one that catalyzes the reaction once the substrate has been bound. In some enzymes, the catalytic site is part of the substrate-binding site; in others, the two sites are structurally as well as functionally distinct. The amino acids that make up the active site do not need to be adjacent in the linear polypeptide sequence; rather, folding of the molecule results in juxtaposition of these amino acids, forming a space in which the substrate sits.

To illustrate how the active site binds a specific substrate and then promotes a chemical change in the bound substrate, we examine the action of *cAMP-dependent protein kinase (cAPK).* This enzyme and other protein kinases, which add a phosphate group to serine, threonine, or tyrosine residues in proteins, are critical for regulating the activity of many cellular proteins. Because the structure of the active site and mechanism of phosphorylation are very similar in all kinases, cAPK can serve as a general model for this important class of enzymes.

As discussed later, an unusual nucleotide called *cAMP* induces dissociation of the inactive tetrameric form of cAPK, releasing two catalytic subunits. To aid in understanding the mechanism of binding and catalysis, we focus here on the 260-residue "kinase core" of each catalytic subunit. The kinase core, which is largely conserved in all protein kinases, is responsible for the binding of ATP and a target peptide, followed by transfer of a phosphate group from ATP to a serine, threonine, or tyrosine in the peptide. The kinase core consists of a large and small domain with an intervening deep cleft; the active site comprises residues located in both domains.

Substrate Binding by Protein Kinases The small domain of the kinase core binds ATP, while the large domain binds the target peptide (Figure 3-23). The structure of the ATP-binding site complements the structure of the nucleotide substrate. The adenine ring of ATP sits snugly at the base of the cleft, which is characterized by a highly conserved sequence, Gly-X-Gly-X-X-Gly. This triad of glycine residues, the "glycine lid," is part of a strand-loop-strand motif that closes over the adenine of ATP and holds it in position. The adenine ring sits in a hydrophobic pocket and is positioned by



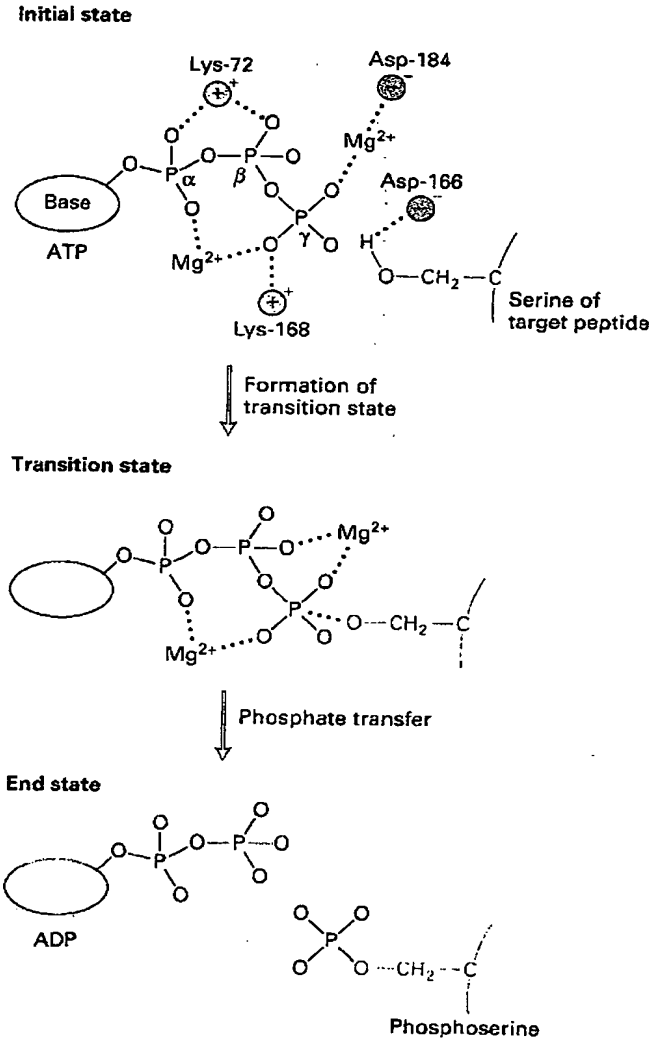
▲ **FIGURE 3-23 Model of the catalytic kinase core of cAMP-dependent protein kinase (cAPK), which is largely conserved in other kinases.** Residues in the small domain position ATP (red) in a deep cleft between the large and small domains of the core. Residues in the large domain bind the target peptide (green). (Courtesy of L. Wu.)

hydrogen bonds and van der Waals attractions with the glycine residues and backbone amide groups. Two invariant residues, lysine at position 72 and aspartic acid at position 184, stabilize the phosphate groups, which protrude from the nucleotide-binding cleft (step 1 in Figure 3-24). Lys-72 bridges to the α and β phosphates of ATP, while the γ-phosphate group is chelated by a Mg^{2+} ion bound to Asp-184.

ATP is a common substrate for all protein kinases, but the sequence of the target peptide varies among different kinases. The peptide sequence recognized by cAPK is Arg-Arg-X-Ser-Y, where X is any amino acid and Y is a hydrophobic amino acid. The portion of the polypeptide chain containing the target serine, threonine, or tyrosine is bound to a shallow groove in the large domain of the kinase core. The peptide specificity of cAPK is conferred by several glutamic acid residues in the large domain, which bind the two arginine residues in the target peptide. Different residues determine the specificity of other protein kinases.

Phosphoryl Transfer by cAPK Figure 3-24 also summarizes the catalytic mechanism of cAPK. Binding of ATP and then the peptide target positions the γ phosphate of ATP near the target serine residue of the peptide. Catalysis takes place in two stages. First, a bond forms between the serine and phosphate group, yielding a pentavalent phosphate transition state. Second, the phosphodiester bond between the β and γ phosphates is broken, yielding ADP and the phosphorylated peptide. Because the phosphate-serine bond is formed on the opposite side of the phosphodiester bond from the β phosphate, this process is called an *in-line mechanism of phosphoryl transfer.* The products, ADP and phosphorylated peptide, are then released from the active site.

During catalysis by cAPK, two "catalytic residues" appear to participate in formation of the transition state. Asp-



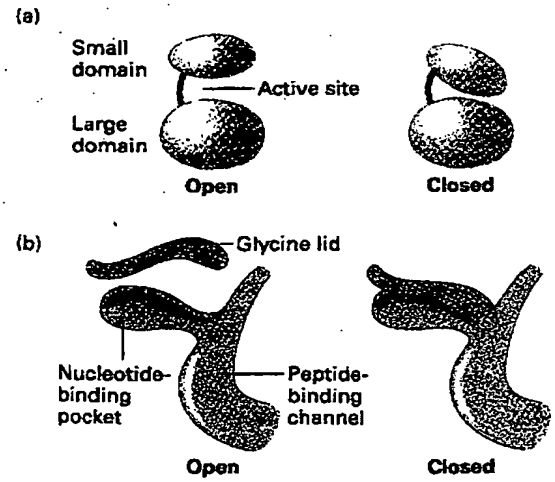
▲ **FIGURE 3-24 The mechanism of phosphorylation by cAMP-dependent protein kinase (cAPK), which catalyzes transfer of a phosphate group from ATP to a serine side chain in a target peptide sequence.** Step 1: Initially, both substrates bind to the active site (see Figure 3-23). Electrons of the phosphate group are delocalized by interactions with lysine residues and Mg^{2+}. Asp166 abstracts a proton from the hydroxyl group of the serine in the bound target peptide. Step 2: A new bond then forms between the serine side-chain oxygen and γ phosphate, yielding a pentavalent transition-state intermediate. Step 3: The phosphoester bond between the β and γ phosphates is broken to form a phosphorylated serine side chain and ADP.

166 is thought to remove a proton from the serine hydroxyl group in the target peptide, while Lys-168 neutralizes the negative charge of the γ phosphate. Then the electrons of the deprotonated serine hydroxyl group are thought to form a bond to the γ phosphorus atom, yielding the pentavalent

transition-state intermediate. The newly created phosphoserine is repelled from the β phosphate of ADP and the catalytic base. The products induce a conformational change in the enzyme, described below, that permits them to diffuse from the active site.

Interactions between residues in the active site of an enzyme and the substrates help stabilize the transition state, thereby allowing more time for the rearrangement of bonds needed to form the products. As explained in Chapter 2, the activation energy is the energy required for formation of the transition state (see Figure 2-27). An enzyme, by virtue of its three-dimensional binding site, reduces the activation energy of a reaction compared with an uncatalyzed reaction involving the same reactants. The ability to bind transition-state intermediates is the one property that distinguishes enzymes from other proteins. If a protein cannot bind a transition-state intermediate, then it cannot catalyze a reaction.

Conformational Changes Induced by Substrate Binding to cAPK The catalytic subunit of cAPK exists in an "open" and "closed" conformation (Figure 3-25a). In the open position, the large and small domains of the kinase core are separated enough that substrate molecules can bind. Once the active site is occupied by substrate, the domains move together into the closed position. This change in tertiary structure, an example of *induced fit*, brings the bound target peptide close enough to the terminal phosphate group of the bound ATP that phosphoryl transfer can occur. After the phosphorylation reaction is completed, the presence of the products causes the domains to rotate to the open position, from which the products are released.



▲ **FIGURE 3-25 Conformational changes in catalytic subunit of cAPK.** Substrate binding causes a rotation of the large and small domains in the catalytic subunit from the open to the closed state (a). This rotation brings the peptide closer to ATP, and also causes the glycine lid to move over the adenine of ATP, thereby trapping the nucleotide in the cleft (b).

The rotation from the open to closed position also causes movement of the short glycine-rich sequence over the ATP-binding cleft in the active site. This small finger of the polypeptide chain, the glycine lid, controls the entry of ATP and release of ADP at the active site. In the open position, ATP can enter and bind to the active site cleft. In the closed position, the glycine-rich sequence moves over the nucleotide and acts as a lid that prevents ATP from leaving (Figure 3-25b). Following phosphoryl transfer, the glycine lid must rotate back to the open position before ADP can be released. Kinetic measurements show that the rate of ADP release is 20-fold slower than that of phosphoryl transfer, reflecting the influence of the glycine lid in cAPK. Mutations in the glycine lid that inhibit its flexibility slow catalysis by cAPK even further. Besides trapping ATP in the binding pocket, the glycine lid prevents water from entering the active site. Water would inhibit the reaction by dampening the charge delocalization steps.

Kinetics of an Enzymatic Reaction Are Described by V_{max} and K_m

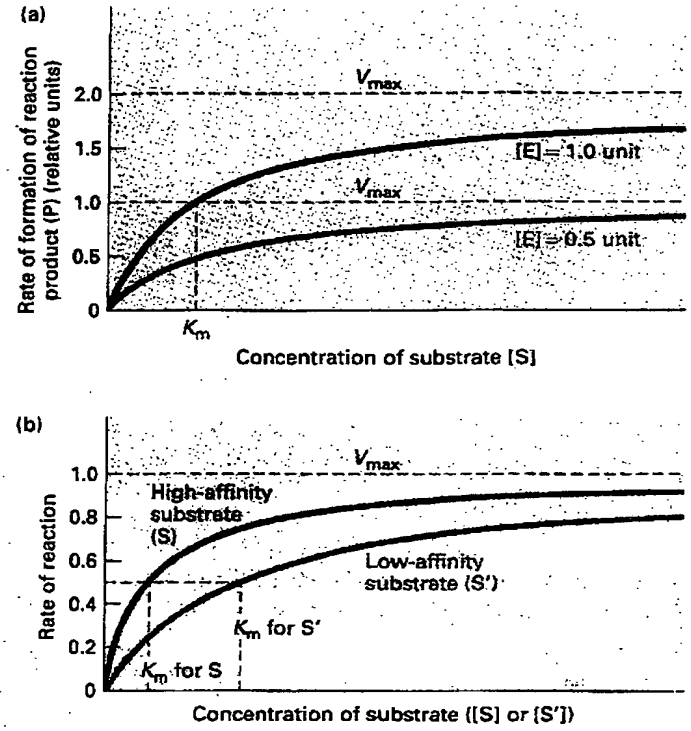
Enzymatic specificity is usually quantified in relative terms; that is, the reaction with a good substrate may occur, for example, 10,000 times faster than it does with a poor substrate. The catalytic action of an enzyme on a given substrate can be described by two parameters: K_m (the Michaelis constant), which measures the affinity of an enzyme for its substrate, and V_{max}, which measures the maximal velocity of the reaction at saturating substrate concentrations. Equations for K_m and V_{max} are most easily derived by considering the simple reaction

$$\text{Substrate} \rightleftharpoons \text{product}$$

in which the rate of product formation v depends on the concentration of substrate, [S], and on the concentration of the enzyme, [E].

For an enzyme with a single catalytic site, Figure 3-26a shows how the rate of product formation depends on [S] when [E] is kept constant. At low concentrations of S, the reaction rate is proportional to [S]. As [S] is increased, the rate does not increase indefinitely in proportion to [S]; rather, it eventually reaches a maximum velocity V_{max}. The value of V_{max} is independent of [S], but is proportional to [E] and to the catalytic constant k_{cat}, which is an intrinsic property of the individual enzyme. Halving [E] reduces the reaction rate at all values of [S] by half. Both V_{max} and K_m for a particular enzyme and substrate can be determined from experimental curves of reaction velocity versus substrate concentration, as illustrated in Figure 3-26.

When interpreting kinetic curves such as those in Figure 3-26, bear in mind that all enzyme-catalyzed reactions include at least three steps: (1) the binding of a substrate (S) to an enzyme (E) to form an enzyme-substrate complex (ES), (2) the conversion of ES to the enzyme-product complex



▲ **FIGURE 3-26 Dependence of the velocity of an enzyme-catalyzed reaction on substrate concentration.** (a) The rates of a hypothetical reaction S ⟶ P at two different concentrations of enzyme [E] as a function of substrate concentration [S]. The [S] that yields a half-maximal reaction rate is the Michaelis constant K_m, a measure of the affinity of E for S. Doubling the concentration of enzyme causes a proportional increase in the reaction rate, so that the maximal velocity V_{max} is doubled; the K_m, however, is unaltered. (b) The rates of the reactions catalyzed by an enzyme with substrate S, for which the enzyme has a high affinity, and with substrate S', for which the enzyme has a low affinity. Note that the V_{max} is the same with both substrates but that K_m is higher for S', the low-affinity substrate.

(EP), and (3) the release of the product (P) from EP, to yield free P:

$$E + S \underset{}{\overset{\text{binding}}{\rightleftharpoons}} ES \xrightarrow{\text{catalysis}} EP \xrightarrow{\text{release}} P + E$$

In the simplest case, when the release of P is very rapid, we can simplify the reaction equation as follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

In this case, the rate of product formation v is equal to $k_{cat} \times [ES]$. Starting from this relationship, we can derive the *Michaelis-Menten equation*

$$v = V_{max}\frac{[S]}{[S] + K_m}$$

where K_m, the Michaelis constant, is defined as $(k_2 + k_{cat})/k_1$. This equation fits the curves shown in Figure 3-26.

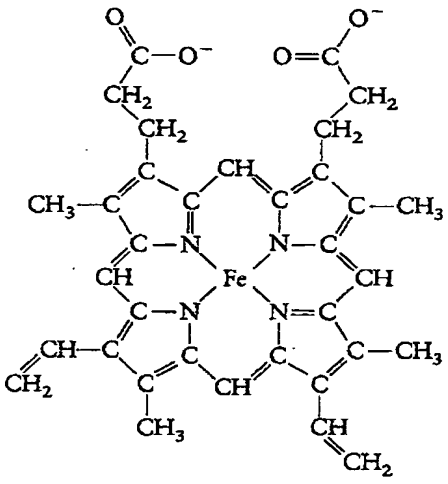
The slowest step in most enzymatic reactions is conversion of the enzyme-substrate complex ES to the free enzyme E and product P. In such cases, k_{cat} is much less than k_2, so that

$$K_m \cong \frac{k_2}{k_1} = K_d$$

where K_d is the dissociation constant for binding of S to E. Thus the parameter K_m describes the affinity of an enzyme for its substrate. The smaller the value of K_m, the more avidly the enzyme can bind the substrate from a dilute solution and the smaller the concentration of substrate needed to reach half-maximal velocity (see Figure 3-26b). The concentrations of the various small molecules in a cell vary widely, as do the K_m values for the different enzymes that act on them. Generally, the intracellular concentration of a substrate is approximately the same as or greater than the K_m value of the enzyme to which it binds.

Many Proteins Contain Tightly Bound Prosthetic Groups

The native conformation and activities of some proteins require the presence of a **prosthetic group**, a small nonpeptide molecule or metal that binds tightly to a protein, keeping the protein in a fixed conformation and participating in binding ligands. For example, each of the four subunits of hemoglobin binds and enfolds a prosthetic group called *heme*, which consists of an iron atom held in a cage by protoporphyrin:



The heme groups are the oxygen-binding components of hemoglobin (see Figure 3-11). Heme is also present in the cytochromes of the electron-transport chain; in this case, it functions to bind electrons. Other electron-transport pro-

teins employ sulfur or flavin as prosthetic groups. In addition to acting as carriers of oxygen or electrons, prosthetic groups can act as antennae. For example, proteins involved in vision or photosynthesis contain retinal or chlorophyll, which absorb energy from sunlight. Prosthetic groups can be linked to proteins noncovalently, as in hemoglobin, or covalently, as in cytochrome.

The activity of numerous enzymes also depends on the presence a prosthetic group, commonly referred to as a coenzyme. Many coenzymes act to lower the activation energy of biochemical reactions by forming a covalent intermediate with a substrate. For instance, the enzyme that converts the amino acid histidine into histamine (a potent dilator of small blood vessels) requires the coenzyme *pyridoxal phosphate*. In this reaction, a covalent bond first forms between histidine and the enzyme-bound pyridoxal phosphate, forming a Schiff base intermediate. Rearrangement of the bonds in this intermediate yields carbon dioxide, which is released, and a second intermediate. This is then hydrolyzed, producing the product, histamine, and regenerating the coenzyme, pyridoxal phosphate.

A Variety of Regulatory Mechanisms Control Protein Function

Most reactions in cells do not occur independently of one another or at a constant rate. Instead, the catalytic activity of enzymes is so regulated that the amount of reaction product is just sufficient to meet the needs of the cell. As a result, the steady-state concentrations of substrates and products will vary depending on cellular conditions. The flow of material in an enzymatic pathway is controlled by several mechanisms, some of which also regulate the functions of nonenzymatic proteins.

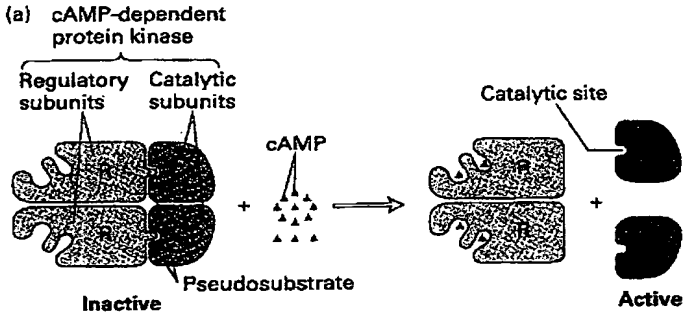
One of the most important mechanisms for regulating protein function entails **allosteric transitions**, changes in the tertiary and/or quaternary structure of a protein induced by binding of a small molecule, which may be an activator, inhibitor, or substrate. Allosteric regulation is particularly prevalent in multimeric (multisubunit) enzymes. Some multimeric enzymes are composed of identical subunits, each containing an active site and, often, a distinct regulatory site. Other enzymes comprise structurally different subunits; in these, active sites and regulatory sites may be located on different subunits.

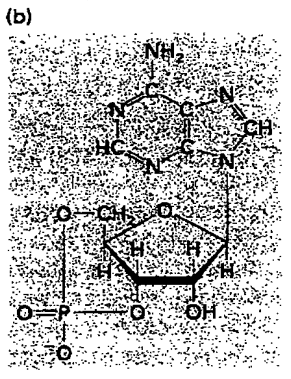
Allosteric Release of Catalytic Subunits As mentioned previously, cAMP-dependent protein kinase (cAPK) exists as an inactive tetrameric protein composed of two catalytic subunits and two regulatory subunits. Each regulatory subunit contains a *pseudosubstrate* sequence that binds to the active site in a catalytic subunit. By blocking substrate binding, the regulatory subunit inhibits the activity of the catalytic subunit. Binding of the allosteric effector molecule cyclic AMP (cAMP) to the regulatory subunit induces a conformational change in the pseudosubstrate sequence so that it no longer can bind the catalytic subunit. Thus the inactive tetramer dissociates into two monomeric active catalytic subunits and a dimeric regulatory subunit (Figure 3-27). As discussed in Chapter 20, binding of various hormones to cell-surface receptors induces a rise in the intracellular concentration of cAMP, leading to activation of cAPK. Once the signaling ceases and the cAMP level decreases, the activity of cAPK is turned off by reassembly of the inactive tetramer.

Allosteric Transition between Active and Inactive States Many multimeric enzymes undergo allosteric transitions that alter the relationship of the subunits to one another but do not cause dissociation as in cAPK. A well-understood enzyme illustrating this mechanism is aspartate transcarbamoylase (ATCase). This bacterial enzyme catalyzes the first step in the pyrimidine biosynthetic pathway:

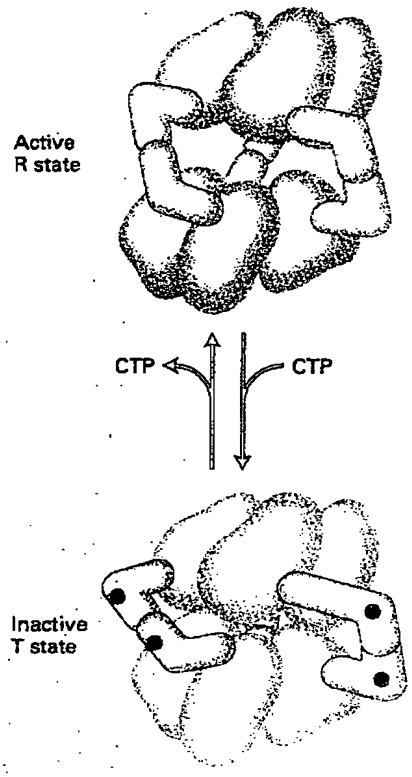
$$\text{Aspartate} + \text{carbamoyl phosphate} \xrightarrow{\text{ATCase}} \underset{N\text{-carbamoylaspartate}}{}$$

ATCase, which is composed of six catalytic subunits and six regulatory subunits, exists in an active R state and inactive T state (Figure 3-28). The equilibrium between these states is shifted toward the inactive T state by binding of cytidine





▲ **FIGURE 3-27 Activation of cAMP-dependent protein kinase (cAPK) by cyclic AMP.** (a) At low concentrations of cAMP, the enzyme exists as an inactive tetramer composed of two regulatory (R) and two catalytic (C) subunits. The tetrameric protein is inactive because the pseudosubstrate sequences on the R subunits block the active sites on the C subunits. Binding of cAMP to the regulatory subunits causes release of the active monomeric catalytic subunits. (b) Structure of cAMP. This unusual nucleotide, which acts as a "second messenger" in many intracellular signaling pathways, controls the activity of many proteins.



▲ **FIGURE 3-28 Allosteric regulation of aspartate transcarbamoylase (ATCase), the initial enzyme in synthesis of pyrimidines.** This enzyme comprises a pair of trimeric catalytic subunits (orange) connected by three pairs of dimeric regulatory subunits (green). Binding of cytidine triphosphate (CTP; the blue dot) to the regulatory subunits causes a conformational transition from the active R state to the inactive T state. The more open conformation of the R state permits substrate binding. Thus an increase in the concentration of CTP, an end product in the pyrimidine pathway, shuts off ATCase, an example of feedback inhibition. [Adapted from B. Mathews and K. E. van Holde, 1996. *Biochemistry*, p. 393.]

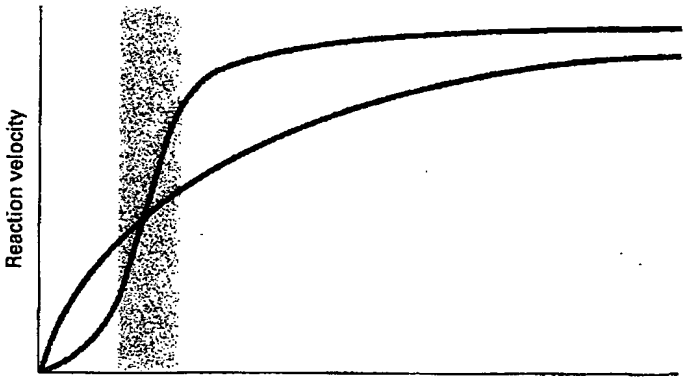
triphosphate (CTP), an end product of the pyrimidine pathway, to the regulatory subunits. Thus CTP is an allosteric inhibitor of ATCase. The CTP-induced allosteric transition in ATCase is an example of *feedback inhibition*, whereby an enzyme that catalyzes an early reaction in a multistep pathway is inhibited by an ultimate product of the pathway. Clearly, this type of regulation prevents accumulation of pyrimidines in excess of what the cell needs for DNA synthesis.

The mechanism of feedback inhibition helps regulate most biosynthetic pathways; that is, the final product of the pathway inhibits the enzyme that catalyzes the first step, thus preventing both production of the intermediate products and unnecessary metabolic activity. Feedback inhibition of enzyme function is reversible. If the concentration of free feedback inhibitor (e.g., CTP) falls, the bound inhibitor dissociates from the regulated enzyme, which then reverts to its active conformation. The binding of a feedback inhibitor to an enzyme and its subsequent release can be described by the equilibrium binding constant K_i, which is similar to the Michaelis constant K_m used to describe substrate binding.

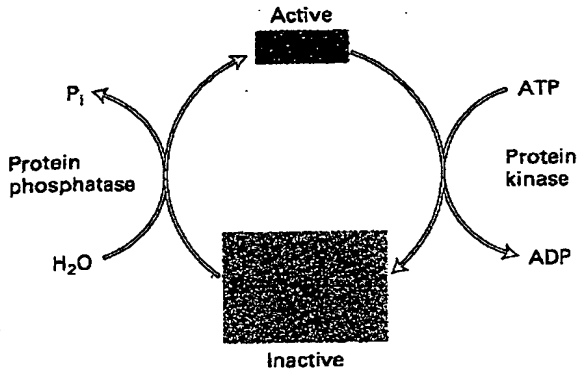
Cooperative Binding of Ligands In many cases, especially when a protein binds several molecules of one ligand, the binding is graded; that is, binding of one ligand molecule affects the binding of subsequent ligand molecules. Such *cooperative allostery*, or cooperative binding, permits many multisubunit proteins to respond more efficiently to small changes in ligand concentration than would otherwise be possible. In positive cooperativity, sequential binding is enhanced; in negative cooperativity, sequential binding is inhibited.

Hemoglobin presents a classic example of positive cooperative binding. Each of the four subunits in hemoglobin can bind one oxygen molecule. Binding of oxygen to one subunit induces a local conformational change whose effect spreads to the other subunits, lowering the K_m for binding of additional oxygen molecules.

Many multimeric enzymes, including aspartate transcarbamoylase (ATCase), also exhibit cooperative binding of substrate. For reactions catalyzed by such enzymes, a plot of reaction velocity versus substrate concentration yields a sigmoidal curve rather than the hyperbolic curve characteristic of enzymes with typical Michaelis-Menten kinetics. As a result of cooperative substrate binding, the maximal enzyme activity (V_{max}) is achieved over a narrow range of substrate concentration (Figure 3-29). Other multimeric enzymes exhibit cooperative binding of an allosteric inhibitor. Because of cooperative allostery, a quite small change in ligand concentration can effectively turn an enzyme on or off.



▲ **FIGURE 3-29 Enzymes with multiple active sites commonly exhibit sigmoidal kinetics (red curve), indicative of cooperative binding of substrates, rather than typical Michaelis-Menten kinetics (blue curve).** Small changes in substrate concentration (pink-shaded region) can effectively switch such allosteric enzymes on or off.

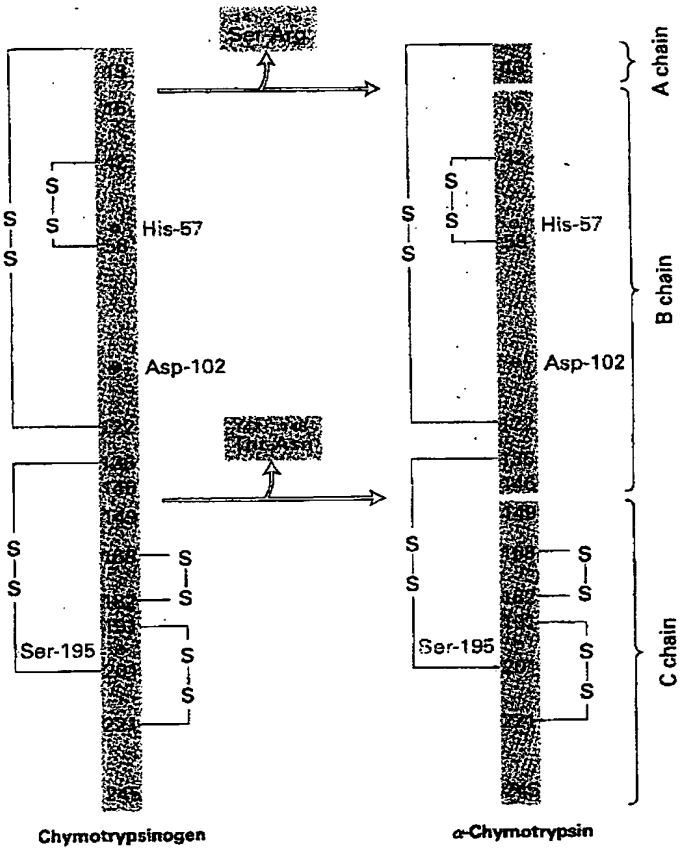


▲ **FIGURE 3-30 Cyclic phosphorylation and dephosphorylation is a common cellular mechanism for regulating protein activity.** In this example, the target protein R (orange) is inactive when phosphorylated and active when dephosphorylated; the opposite pattern occurs in some proteins.

Cyclic Protein Phosphorylation and Dephosphorylation As noted earlier, one of the most common mechanisms for regulating protein activity is the addition and removal of phosphate groups from serine, threonine, or tyrosine residues. Protein kinases catalyze phosphorylation, and phosphatases catalyze dephosphorylation. Although both reactions are essentially irreversible, the counteracting activities of kinases and phosphatases provide cells with a "switch" that can turn on or turn off the function of various proteins (Figure 3-30). Phosphorylation changes a protein's charge and generally leads to a conformational change; these effects can significantly alter ligand binding by a protein and its activity.

Nearly 3 percent of all yeast proteins are protein kinases or phosphatases, reflecting the importance of phosphorylation and dephosphorylation reactions even in simple cells. These enzymes target all classes of proteins including structural proteins, enzymes, membrane channels, and signaling molecules. In later chapters, we will encounter many examples of important cellular functions that are controlled by phosphorylation and dephosphorylation of specific proteins.

Proteolytic Activation The regulatory mechanisms discussed so far can act like switches, turning proteins on and off. Regulation of some enzymes involves the irreversible activation of an inactive form, commonly at the site where the active enzyme is needed. Good examples of such enzymes are the digestive proteases trypsin and chymotrypsin, which are synthesized in the pancreas and secreted into the small intestine as inactive precursors, or *zymogens*, called *trypsinogen* and *chymotrypsinogen*, respectively. In the protease-rich environment of the small intestine, the zymogens are converted to the active enzymes, which then begin to hydrolyze the peptide bonds of ingested proteins. As shown in Figure 3-31, two irreversible proteolytic cleavages of chymotrypsinogen yield active chymotrypsin. The delay in activation of these proteases until they reach the intestine prevents them from digesting the pancreatic tissue in which they are made.



▲ **FIGURE 3-31 A linear representation of the conversion of chymotrypsinogen into chymotrypsin by the excision of two dipeptides.** These reactions yield three separate chains (A, B, and C), which are covalently linked by disulfide bonds (yellow) in the active enzyme. In the folded, native conformation of chymotrypsin, histidine 57, aspartate 102, and serine 195 are located in the active site.

Other Regulatory Mechanisms The activities of enzymes are extensively regulated in order that the numerous enzymes in a cell can work together harmoniously. All metabolic pathways are closely controlled at all times. Synthetic reactions occur when the products of these reactions are needed; degradative reactions occur when molecules must be broken down. All the regulatory mechanisms described above affect enzymes locally at their site of action.

Regulation of cellular processes, however, involves more than simply turning enzymes on and off. Some regulation is accomplished by keeping enzymes in compartments where the delivery of substrate or exit of product is controlled. In many cases, the compartments are organelles, such as the mitochondria, nuclei, or lysosomes. Compartmentation permits competing reactions to occur simultaneously in different

parts of a cell. In addition to compartmentation, cellular processes are regulated by enzyme synthesis and destruction. Often enzymes are synthesized at low rates when the cell has no need for their activities; however, upon increased demands by the cell (for instance, appearance of substrate), new enzyme is synthesized. Later, the pool of enzyme is lowered when levels of substrate decrease or the cell becomes inactive.

SUMMARY Functional Design of Proteins

- The function of nearly all proteins depends on their ability to bind other molecules (ligands). Ligand-binding sites on proteins and the corresponding ligands are chemically and topologically complementary. The affinity of a protein for a particular ligand refers to the strength of binding; its specificity, to the restriction of binding to one or a few preferred ligands.
- Enzymes are catalytic proteins that accelerate the rate of cellular reactions by lowering the activation energy and stabilizing transition-state intermediates.
- Enzyme active sites comprise two functional parts: a substrate-binding region and a catalytic region. The amino acids composing the active site are not necessarily adjacent in the amino acid sequence, but are brought into proximity in the native conformation.
- The kinetics of many enzymes are described by the Michaelis-Menten equation. From plots of reaction rate versus substrate concentration, two characteristic parameters of an enzyme can be determined: the Michaelis constant K_m, a measure of the enzyme's affinity for substrate, and the maximal velocity V_{max} (see Figure 3-26).
- Many multimeric enzymes and other proteins exhibit allostery. In this phenomenon, binding of one ligand molecule (a substrate, activator, or inhibitor) induces a conformational change, or allosteric transition, that alters the protein's activity or affinity for other ligands.
- In multimeric proteins that bind multiple ligands, binding of one ligand molecule may increase or decrease the binding affinity for subsequent ligand molecules. Enzymes that cooperatively bind substrates exhibit sigmoidal kinetics (see Figure 3-29).
- Allosteric mechanisms can act like switches, turning protein activity on and off. Cyclic phosphorylation and dephosphorylation of amino acid side chains can have the same regulatory effect. Proteolytic cleavage irreversibly converts inactive zymogens into active enzymes.

3.4 Membrane Proteins

As we've seen, all antibodies have a similar structure and function; enzymes are structurally varied, but all have a catalytic function. In contrast, although all membrane proteins are located at the membrane, they otherwise are both structurally and functionally diverse. As we noted in Chapter 2 and discuss in more detail in Chapter 5, every biological membrane has the same basic phospholipid bilayer structure. Associated with each membrane is a set of membrane proteins that enables the membrane to carry out its distinctive activities (Figure 3-32). The complement of proteins attached to a membrane varies depending on cell type and subcellular location.

Some proteins are bound only to the membrane surface, whereas others have one region buried within the membrane and domains on one or both sides of it. Protein domains on the extracellular membrane surface are generally involved in cell-cell signaling or interactions. Domains within the membrane, particularly those that form channels and pores, move molecules across the membrane. Domains lying along the cytosolic face of the membrane have a wide range of functions, from anchoring cytoskeletal proteins to the membrane to triggering intracellular signaling pathways. In many cases, the function of a membrane protein and the topology of its polypeptide chain in the membrane can be predicted based on its homology with another, well-characterized protein. In this section, we examine the characteristic structural features of membrane proteins and some of their basic functions. More complete characterization of the structure and function of various types of membrane proteins is presented in several later chapters. The synthesis and processing of membrane proteins are discussed in Chapter 17.

Proteins Interact with Membranes in Different Ways

Membrane proteins can be classified into two broad categories—integral (intrinsic) and peripheral (extrinsic)—based on the nature of the membrane-protein interactions (see Figure 3-32). Most biomembranes contain both types of membrane proteins.

Integral membrane proteins, also called *intrinsic proteins,* have one or more segments that are embedded in the phospholipid bilayer. Most integral proteins contain residues with hydrophobic side chains that interact with fatty acyl groups of the membrane phospholipids, thus anchoring the protein to the membrane. Most integral proteins span the entire phospholipid bilayer. These *transmembrane* proteins contain one or more membrane-spanning domains as well as domains, from four to several hundred residues long, extending into the aqueous medium on each side of the bilayer. In all the transmembrane proteins examined to date, the membrane-spanning domains are α helices or multiple β strands. In contrast, some integral proteins are anchored to one of the membrane leaflets by covalently bound fatty acids, as discussed later. In these proteins, the bound fatty acid is embedded in the membrane, but the polypeptide chain does not enter the phospholipid bilayer.

Peripheral membrane proteins, or extrinsic proteins, do not interact with the hydrophobic core of the phospholipid

Anal. Calcd. for $C_{10}H_{18}O_9$: C, 42.53; H, 6.42. Found: C, 42.5; H, 6.4.

Iodimetric titration gave the expected equivalent value for a disaccharide and on hydrolysis the final reducing value and specific rotation were those calculated for D-xylose. On acetylation of the disaccharide, there was produced the same crystalline hexaacetyl xylobiose as reported by Bachrach and Whistler.[2] Thus, the structure of the disaccharide is 4-(β-D-xylopyranosyl)-β-D-xylopyranose.

In another instance a 2% xylan solution in 42% hydrochloric acid solution was hydrolyzed to 50% of completion. It was neutralized and chromatographically separated as before. After washing the column with water and 5% ethanol, a trisaccharide fraction was removed with 9% ethanol. The concentrated sirup was dissolved in a small amount of warm water and hot absolute ethanol was added to produce a solution of 80–85% alcohol concentration. Upon cooling, crystallization occurred. The yield was 8.0% of the original xylan; m.p. 205–206°; $[\alpha]_D^{25}$ 46.96 (1.06% in water).

Anal. Calcd. for $C_{15}H_{26}O_{13}$: C, 43.48; H, 6.33. Found: C, 43.4; H, 6.4.

Iodimetric titration gave the expected value for a trisaccharide and on hydrolysis the reducing value and specific rotation were those calculated for D-xylose.

(2) J. Bachrach and R. L. Whistler, paper presented before the Division of Sugar Chemistry, 118th meeting of American Chemical Society, Atlantic City, 1949.

DEPARTMENT OF AGRICULTURAL CHEMISTRY
PURDUE UNIVERSITY ROY L. WHISTLER
WEST LAFAYETTE, INDIANA C. C. TU

RECEIVED FEBRUARY 3, 1951

ADRENAL CONVERSION OF C^{14} LABELED CHOLESTEROL AND ACETATE TO ADRENAL CORTICAL HORMONES[1]

Sir:

It previously has been demonstrated that beef adrenals, perfused with blood containing added ACTH, synthesize and release into the perfusion medium a mixture of corticosteroids, the principal components of which are 17-hydroxycorticosterone (I) and corticosterone (II).[2,3] We wish to report that when similar experiments are carried out in the presence of either C^{14} labeled acetate or cholesterol, the I and II isolated from adrenal perfusates are radioactive, and have approximately the same number of counts per mg. per min. (c.m.m.) as determined under identical conditions.

Groups of 5 glands were perfused in parallel from a manifold with 1 liter of homologous citrated blood containing 25 mg. of ACTH (Armour) for four hours, the perfusate being recycled through the glands. The corticosteroids were extracted from perfusates with activated carbon (Darco G-60),[4] and fractionated by paper partition chromatography. The compounds were characterized by the method of mixed chromatograms,[5] both of the free steroids and of their esters, and by measuring the chromogen produced by H_2SO_4.[6] All counts were made using a thin-window Geiger counter with 0.1–0.7 mg. samples (diluted with non-isotopic compound when necessary) plated as a thin film. Ten milligrams of carboxyl labeled sodium acetate having radioactivity of 5.8×10^6 c.m.m. was added to the medium at the initiation of the perfusion. I and II were isolated in **3.0** and **1.0** mg. amounts, and had activity of 319 and 305 c.m.m. respectively, in one experiment; in a second similar experiment, the c.m.m. for each were 219 and 208 and the total amounts isolated were **4.5** and **1.5** mg. for I and II, respectively. The counts were made with 0.09 to 0.13 mg. samples; with our technique the c.m.m. of I or II remains constant in the range 0.09 to 0.7 mg.

A similar perfusion of cholesterol labeled in position 3 with C^{14} prepared from radio-cholestenone[7] by Drs. Schwenk, Gut and Belisle[8] was conducted in which 90 mg. of radiocholesterol (300 c.m.m.) was used. I and II were isolated in **1.0** and **0.4** mg. amounts and had activities of 25 and 18 c.m.m., respectively.

The data of Table I indicate that the radioactivity is a property of the compounds isolated since (a) rechromatography on paper and (b) the preparation of two derivatives led to no significant alteration of the specific activity. It is recognized that the method of mixed chromatograms of the free compounds and their esters plus determination of the H_2SO_4 chromogen does not constitute a classical characterization of I and II. In our experience, however, no substance proved to be homogeneous by this method, has failed to meet classical criteria of purity and composition.

TABLE I

The specific activities of cortical hormones and their derivatives isolated from an adrenal perfusion experiment with $CH_2C^{14}OONa$.

		mg.	c.m.m.
1	Free 17-hydroxycorticosterone (I)[a]	0.125	319
2	I after rechromatography	.115	340
3	I acetate[b]	.130	362
4	I propionate[b]	.098	332
1	Free corticosterone (II)[a]	.130	305
2	II after rechromatography[a]	.090	326
3	II acetate[b]	.123	294
4	II propionate[b]	.110	342

[a] Isolated from paper following partition chromatography using the propylene glycol–toluene system. [b] Isolated from paper following partition chromatography using the formamide–benzene system. The samples were then rechromatographed on paper.

These data indicate that both acetate and cholesterol can be transformed by the isolated adrenal

(1) Aided by United States Public Health Service Grant GG-2742 and G. D. Searle and Company.

(2) Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus, *Recent Progress in Hormone Research*, in press.

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gland into adrenocortical steroids. While cholesterol appears to be a more efficient precursor than acetate, further experiments are necessary to accurately define the percentage conversion with both precursors. Since in adrenal slices cholesterol has been shown to arise from acetate condensation[9] it is not inconceivable that cholesterol may be an intermediary in the reactions leading to corticosteroid synthesis from acetate. We hope in further studies to determine whether cholesterol is an obligatory intermediate in steroidogenesis.

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BOOK REVIEWS

Principles of Ionic Organic Reactions. By ELLIOT R. ALEXANDER, Assistant Professor of Chemistry in the University of Illinois. John Wiley and Sons, Inc., 440 Fourth Avenue, New York 16, N. Y. 1950. viii + 318 pp. 15.5 × 23.5 cm. Price, $5.50.

This book applies ionic principles in presenting the mechanisms of those organic reactions where the ionic concept has been established or appears very likely. The treatment is entirely from the point of view of the organic chemist and actual kinetic data and mathematics are omitted. The author has not merely presented reaction explanation but has also presented the important data leading to the validity of the ionic mechanisms. The documentation is adequate but not exhaustive.

The book will serve admirably as a primary textbook for an advanced course in organic reaction mechanisms to follow a more orthodox advanced chemistry course, or as a supplementary book for a comprehensive advanced organic course. The research organic chemist whose formal schooling was completed say ten years ago will find this an indispensable means of mastering the newer concepts.

The style and treatment are, for the most part, quite clear although more explanation of the symbolism employed might be suggested. More detail would be helpful in explaining the actual physical nature of carbonium ions and the sequence of events leading up to their transitory existence. Conspicuous omissions include ionic oxidation reactions and the application of the transition state concept to ionic processes.

The complete objectivity of the book impresses this reviewer. Although Dr. Alexander is a firm believer in ions he accepts them, applies them, and submits the evidence without any apparent attempt to crusade for them. This is a healthy attitude in a book of this type; one regrets that the same approach has not yet been used in free radical books. The book is excellent and highly recommended.

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Technique of Organic Chemistry. Volume I, Part II. **Physical Methods of Organic Chemistry.** Edited by ARNOLD WEISSBERGER. Interscience Publishers, Inc., 250 Fifth Avenue, New York 1, N. Y. 1949. xi + 1024 pp. 15.5 × 23 cm. Price, $12.50.

The chapter headings, and the authors, are as follows: X-Ray Diffraction, by I. Fankuchen; Electron Diffraction, by L. O. Brockway; Refractometry, by N. Bauer and K. Fajans; Spectroscopy and Spectrophotometry by W. West; Colorimetry, Photometric Analysis, Fluorimetry and Turbidimetry, by W. West; Polarimetry, by W. Heller; Determination of Dipole Moments, by C. P. Smyth; Conductometry, by T. Shedlovsky; Electrophoresis, by D. H. Moore; Potentiometry, by L. Michaelis; Polarography, by O. H. Müller; Determination of Magnetic Susceptibility, by L. Michaelis; Determination of Radioactivity, by W. F. Bale and J. F. Bonner, Jr.; Mass Spectrometry, by D. W. Stewart.

This book is necessarily of encyclopedic proportions and character, and like other encyclopedias it must suffer from obsolescence setting in before the ink is dry. Indeed, if a book of this nature is to be any good, its publication must accelerate its own obsolescence, for if the authors are conscientious in pointing out the pitfalls and the weaknesses of existing procedures and instruments this must in turn stimulate new advances. By this criterion the book is not a very good one. On the whole it shows little dissatisfaction with present-day instrumentation.

The authors have been faced with the necessity of compromising between theory and experimental techniques; for the most part they have elected to present an adequate treatment of fundamental principles at the expense of detailed experimental procedures. The book is thus in no sense a working manual but it is a comprehensive and authoritative reference source of material not otherwise readily available to the analyst.

If any one chapter excels in excellence and thoroughness, the reviewer would choose the discussion of polarimetry by W. Heller. Granted the advantage of dealing with a quiescent, maturely developed field, this presentation is worthy of special comment for the abundant illustrations, the preciseness of its formulation, and for the wealth of information it contains.

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Fortschritte der Alkaloidchemie Seit 1933. Scientia Chimica, Band 2. By HANS-G. BOIT, Chemisches Institute der Universität, Berlin. Akademie-Verlag G. m. b. H., Presseabteilung, Schiffbauerdamm 19, Berlin NW 7, Germany. 1950. xxxii + 425 pp. 18 × 25 cm. Price, paper, 49 DM, bound, 53 DM.

A progress report is one of the devices which a busy worker in one field can use to keep abreast with advances in another field. The present monograph serves this purpose excellently. It does not present the extensive background in alkaloid chemistry which preceded 1933, the year chosen for the start of the report. It does attempt to supplement some of the excellent reviews available at that time, such as Winterstein-Trier, with a broad account of the work which has been completed in the intervening years. In spite of this, the book is self-contained. The author follows the practice of giving sufficient information in each discussion to pick up the thread of the argument.

The major portion of the book is devoted to a discussion of the chemistry of alkaloids whose structures are either completely elucidated or tentatively assigned. These are

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DETERMINANTS OF SEX HORMONE LEVELS IN MEN AS USEFUL INDICES IN HORMONE-RELATED DISORDERS

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Abstract—Because the determinants of serum sex hormone levels in men have been infrequently studied, we investigated the relation of several personal characteristics to serum levels of testosterone (T), dihydrotestosterone (DHT), estrone (E1), estradiol (E2) and sex hormone-binding globulin (SHBG) among 98 Japanese American men in Hawaii, aged 52–74. The SHBG levels and T/(E1 + E2) ratios decreased progressively with increasing body mass index. The SHBG levels were also inversely associated with hematocrit levels. Serum androgen and estrogen levels did not correlate with smoking, alcohol intake, serum cholesterol, serum uric acid and blood pressure. Some of the associations observed in the present study may be implicated in the etiology of hormone-related neoplasms in men.

Sex hormones Body mass index Smoking Alcohol

INTRODUCTION

Studies have suggested that sex hormones may play an important role in the pathogenesis of human cancer [1]. Possible sites include breast, ovary, endometrium, thyroid, testis and prostate [1]. If determinants of hormone levels are identified, controlling these factors may lead to primary prevention of hormone-related cancers. Moreover, such factors would be useful indices in future epidemiologic studies of hormone-related diseases.

In men, androgens are suspected to have a role in the etiology of prostate cancer. They are required for the growth and function of the prostate gland [2] and the administration of testosterone can produce prostatic adenocarcinoma in experimental animals [3–5]. Eunuchs have not been observed clinically with prostate cancer [6] and castration or estrogen therapy is helpful against prostate cancer [7]. However, studies of the serum or plasma hormone levels have produced equivocal results with respect to prostate cancer risk [6]. This may be partly due to a limited understanding of the epidemiology of serum sex hormone levels in men. In comparison, a number of studies have identified various determinants of sex hormonal levels in women [8–11]. They include reproductive and anthropometric factors, which have been associated with hormone-related cancers in women [12–14].

Because it is likely that anthropometric and other characteristics are related to sex hormone levels in men, as observed in women, this report compares sex hormone levels in men with sev-

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eral of their personal characteristics in a population-based study.

METHODS

The study subjects were 98 men, aged 52–74, who were originally selected as controls in a nested case–control study of prostate cancer. They were matched to 98 prostate cancer incident cases by sex and year of examination. Details in the method of selection of these subjects were described elsewhere [15]. The original cohort, which consisted of 8006 men of Japanese ancestry, born from 1900 to 1919, and residing on the Hawaiian island of Oahu, were examined by the Honolulu Heart Program from 1965 to 1968. Approximately 6 years later, from 1971 to 1975, 6860 of these men returned for another round of examinations. At that time, a non-fasting venous blood sample was drawn between 7 a.m. and 7 p.m. The sera were stored at −75°C. The frozen sera, which had never been thawed before, were sent in dry ice to Los Angeles for analysis. Testosterone levels were measured by the method of Anderson *et al.* [16]. Radioimmunoassay procedures were used to determine the serum levels of dihydrotestosterone [17]. Estrone and estradiol were measured by the method of DeVane *et al.* [18]. Sex hormone-binding globulin activity was determined by the selective ammonium sulfate precipitation technique of Rosner [19], using tritiated dihydrotestosterone as ligand.

The following variables were collected at the time the blood sample was obtained: cigarette smoking history, alcohol intake (oz./mo), body mass index (weight (kg)/height (m)^2), age at first marriage and systolic and diastolic blood pressure, serum total cholesterol levels, serum uric acid levels and hematocrit. Data on marital status and number of children were collected at the time of the 1965–68 examination.

Due to skewed serum hormone distributions, Spearman's correlation coefficients were calculated between each hormone measurement and the personal characteristics [20]. Analysis of covariance [21] was used to calculate time (of the day that the specimen was drawn) adjusted mean hormone levels by age group after logarithmic transformation. Logarithmic transformation was also used in calculating time and age adjusted mean hormone levels by tertiles of specific personal characteristics. Multiple regression analysis [22] was performed to test for trend in logarithmic transformations of hormone levels according to specific personal characteristics while adjusting for covariates.

RESULTS

Table 1 shows time-adjusted hormone levels by age group (<60, 60–64, 65–69, 70+). There was no linear trend with advancing age in each of the hormone levels.

Next, Spearman's correlation coefficients were calculated between hormone measurements and personal characteristics, as shown in Table 2. Serum testosterone levels were inversely associated with time of blood collection, i.e. high in the morning and low in the evening. Serum sex hormone-binding globulin activity was inversely associated with body mass index and hematocrit levels. The testosterone/dihydrotestosterone ratio was inversely related to number of children and the testosterone/(estrone + estradiol) ratio was inversely associated with body mass index.

The significant findings in Table 2 are presented in Table 3 according to the mean hormonal levels by tertiles of the relevant factors, adjusted for age and time of collection. The strength of the association between the testosterone/dihydrotestosterone ratio and number of

Table 1. Time-adjusted mean hormone levels and their standard errors (SE) by age group

Serum hormone	Age group 52–59	60–64	65–69	70–74	Total
Testosterone (T)*	3845.1 ± 330.7	3570.8 ± 355.9	4353.3 ± 306.8	3581.1 ± 472.9	3944.8 ± 177.3
Dihydrotestosterone (DHT)*	379.9 ± 32.1	380.4 ± 37.2	459.0 ± 31.7	406.9 ± 52.7	413.2 ± 17.9
Estrone (E1)*	30.4 ± 2.2	30.6 ± 2.6	28.0 ± 1.7	29.6 ± 3.4	29.4 ± 1.1
Estradiol (E2)*	17.7 ± 1.1	18.3 ± 1.4	18.3 ± 1.0	17.2 ± 1.7	18.0 ± 0.6
Sex hormone binding globulin†	3.5 ± 0.2	2.9 ± 0.2	4.4 ± 0.3	3.8 ± 0.4	3.7 ± 0.1
T/DHT	10.1 ± 0.7	9.4 ± 0.8	9.5 ± 0.5	8.8 ± 0.9	9.5 ± 0.3
T/(E1 + E2)	79.1 ± 7.7	72.1 ± 8.2	92.3 ± 7.4	75.0 ± 11.3	82.0 ± 4.1
No. of subjects	27	20	39	12	98

*pg/ml.

Table 2. Spear

Characteristics
Age at exam
Hour of day
Body mass index
Smoking status†
No. of cigarette/da
Alcohol intake (oz/
Marital status‡
Age at first marria
No. of children
Hematocrit
Serum cholesterol
Serum uric acid
Systolic blood pres

T: testosterone; DH
*$p < 0.01$; **$p < 0.0$
†Never = 1; past =
‡Married/remarried

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Table 3. Age- and
values by tertile

Hormones	Char
SHBG‡	Body m
	<21.
	21.6–
	≥24.
	Hematc
	≤41
	42–4
	≥45
T/DHT	No. of
	0–1
	2–3
	≥4
T/E	Body m
	<21.
	21.6–
	≥24.

SHBG: sex hormo
DHT: dihydrote
*$p < 0.01$, compare
†Based on multiple

Table 2. Spearman correlation coefficients between serum hormone levels and personal characteristics in men

Characteristics	T	DHT	E1	E2	SHBG	T/DHT	T/E
Age at exam	0.10	0.16	−0.01	0.05	0.21	−0.07	0.06
Hour of day	−0.27*	−0.12	−0.16	−0.14	0.07	−0.15	−0.08
Body mass index	−0.18	−0.22	0.13	0.22	−0.37***	0.04	−0.32**
Smoking status†	−0.06	−0.02	0.13	−0.05	−0.02	−0.08	−0.12
No. of cigarette/day	−0.05	−0.05	0.15	−0.08	0.01	−0.05	−0.09
Alcohol intake (oz/mo)	0.05	0.09	0.09	−0.08	0.12	−0.07	0.05
Marital status‡	−0.16	−0.11	−0.15	−0.21	−0.20	−0.11	−0.02
Age at first marriage	0.00	−0.12	−0.16	0.14	0.10	0.19	0.06
No. of children	0.04	0.24	0.04	−0.16	0.22	−0.25*	0.06
Hematocrit	0.00	0.02	0.21	0.08	−0.26*	−0.06	−0.12
Serum cholesterol	0.09	0.16	0.06	0.04	−0.06	−0.10	0.03
Serum uric acid	0.06	0.12	0.19	0.20	0.03	−0.09	−0.09
Systolic blood pressure	−0.12	−0.04	0.13	0.06	−0.09	−0.05	−0.20

T: testosterone; DHT: dihydrotestosterone; E1: estrone; E2: estradiol; E: E1 + E2; SHBG: sex hormone binding globulin.
*$p < 0.01$; **$p < 0.005$; ***$p < 0.001$.
†Never = 1; past = 2; current smokers = 3.
‡Married/remarried = 1; others = 0.

children was reduced, but the other associations remained strong.

DISCUSSION

It has been suggested that sex hormones play an important role in the pathogenesis of cancer among both men and women [1]. Although the association between each type of sex hormone and cancer risk has been rather vague among men compared with women [6], this investigation identified several possible determinants of hormone levels in men. The testosterone (estrone + estradiol) ratio was inversely associated with body mass index in this study. Earlier studies also found decreased testosterone levels [23–28] and increased estrogen levels [23–25] in obese men. Approximately 75–90% of the estrogens in the plasma of young healthy men is derived from the conversion of testosterone and its precursor (androstenedione) in adipose tissue [2]. This could explain the observed associations. An inverse association between obesity and sex hormone binding globulin, as reported previously [24, 25, 27–30], was also supported by the present study.

It is uncertain if obesity increases prostate cancer risk. Several studies have found that an increase in body mass index or relative weight was positively associated with prostate cancer [31–33]. However, other investigators could not confirm this finding [34–36].

The present study had several methodological limitations. Because of the narrow age range of the study subjects (52–74 years old), the findings do not necessarily apply to younger adults. The statistical significance of some associations could be due to chance because of the multiple comparisons made, and the study design limits our ability to establish causal or temporal relationships.

Changes in sex hormone levels with age are still unclear. Several earlier studies showed that there is an increase in estrogen levels [37] and a reduction in testosterone levels [26, 37, 38] with advancing age. Other studies found a slight increase instead of decrease in plasma testosterone levels with age [29, 39]. Coffey, in turn, noted that the average testosterone concentration in adult male plasma was not greatly related to between 25 and 70 years [2]. We also observed no clear association between age and hormone levels in men.

There have been few consistent findings on the influences of cigarette smoking, alcohol in-

Table 3. Age- and time-adjusted mean levels of hormonal values by tertiles of specific personal characteristics

Hormones	Characteristics	No.	Mean	Trend p†
SHBG‡	Body mass index			
	<21.6	32	4.3	0.004
	21.6–24.3	34	3.5	
	≥24.4	32	3.4*	
	Hematocrit (%)			
	≤41	30	4.3	0.006
	42–44	35	3.6	
	≥45	33	3.4	
T/DHT	No. of children			
	0–1	20	11.3	0.046
	2–3	41	9.2	
	≥4	37	9.1	
T/E	Body mass index			
	<21.6	32	94.9	0.011
	21.6–24.3	34	75.2	
	≥24.4	32	77.7	

SHBG: sex hormone binding globulin; T: testosterone; DHT: dihydrotestosterone; E: estrone + estradiol.
*$p < 0.01$, compared with the lowest tertile.
†Based on multiple regression analysis.

levels in men. A study among university students found that plasma testosterone concentration was positively correlated with cigarette smoking [40], but another study revealed that the association was indirect through age [26]. Plasma estradiol levels among sedentary middle-aged men were positively correlated with number of cigarettes per day [41], whereas female cigarette smokers were found to have a decreased level of urinary estrogen concentration in the luteal phase [42]. No effect of chronic alcohol consumption on serum testosterone levels was found in two studies [26, 29] and a suppressive effect was observed in another study [41]. In the present study, neither smoking nor alcohol was associated with either androgen or estrogen levels.

It is known that androgens stimulate erythropoiesis [2]. Although the present study did not find a positive association between serum androgen levels and hematocrit, the serum sex hormone-binding globulin activity was inversely associated with hematocrit levels. Lower levels of SHBG could result in a higher level of free testosterone, not bound to globulin molecules. As a consequence, the free fraction of testosterone could be positively associated with hematocrit levels, but we were unable to measure the unbound portion of testosterone in the serum.

It has been suggested that sex hormones play a significant role in determining several cardiovascular risk factors, i.e. serum lipid and uric acid levels and blood pressure. Hypertension, hypercholesteremia and hyperuricemia are more common in men than in premenopausal women [43]. Our results, based on multiple regression analyses, did not find any significant associations between sex hormone levels in men and serum cholesterol, serum uric acid and blood pressure.

Acknowledgement—This work was supported in part by Grant ROI CA 33644 from the National Cancer Institute, National Institutes of Health, Bethesda, Maryland.

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